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Identification of Extremely Reactive γ-Ketoaldehydes (Isolevuglandins) as Products of the Isoprostane Pathway and Characterization of Their Lysyl Protein Adducts*

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Isoprostanes are prostaglandin-like compounds produced by non-enzymatic peroxidation of arachidonic acid. The cyclooxygenase-derived endoperoxide, prostaglandin H_2, can undergo rearrangement to highly reactive γ-ketoaldehyde secoprostanoids (levuglandin E_2 and D_2). We explored whether isoprostane endoperoxide intermediates also rearrange to levuglandin-like compounds (isolevuglandins). Formation of a series of isolevuglandins during oxidation of arachidonic acid *in vitro* was established utilizing a number of mass spectrometric analyses. However, these compounds could not be detected in free form in protein-containing biological systems, which we hypothesized was due to extremely rapid adduction to amines. This was supported by the finding that >60% of levuglandin E_2 adducted to albumin within 20 s, whereas ~50% of 4-hydroxynonenal still remained unadducted after 1 h. By utilizing electrospray tandem mass spectrometry, we established that these compounds form oxidized pyrrole adducts (lactams and hydroxylactams) with lysine. Formation of isolevuglandin-lysine adducts on apolipoprotein B was readily detected during oxidation of low density lipoprotein following enzymatic digestion of the protein to single amino acids. These studies identify a novel series of extremely reactive products of the isoprostane pathway that rapidly form covalent adducts with lysine residues on proteins. This provides the basis to explore the formation of isolevuglandins *in vivo* to investigate the potential biological ramifications of their formation in settings of oxidant injury.

A role for free radicals has been implicated in the pathogenesis of a wide variety of human diseases including atherosclerosis, cancer, and neurodegenerative diseases (1). Unsaturated fatty acids are a major target of free radical attack leading to lipid peroxidation. A variety of compounds is generated as products of free radical-induced lipid peroxidation including reactive aldehyde species, such as 4-hydroxynonenal (4-HNE)[2] and malondialdehyde (MDA). These reactive aldehydes are considered important mediators of oxidant injury due to their ability to covalently modify proteins and DNA, which can disrupt important cellular functions and can cause mutations (2). Furthermore, adduction of aldehydes to apolipoprotein B in LDL has been strongly implicated in the mechanism by which LDL is converted to an atherogenic form that is taken up by macrophages, eventuating in the formation of foam cells (3). Previously we reported the formation of prostaglandin (PG)-like compounds *in vivo*, termed isoprostanes (IsoPs), by free radical-induced peroxidation of arachidonic acid (4). Analogous to the cyclooxygenase enzymatic pathway, intermediates in the IsoP pathway are PGH_2-like bicyclic endoperoxides. In aqueous media, PGH_2 is unstable and undergoes rearrangement with a $t_{1/2}$ of approximately 5 min at 37 °C (5). Originally, it was shown PGH_2 undergoes rearrangement to form PGE_2 and PGD_2 (6). More recently, Salomon and colleagues (5) demonstrated that PGH_2 also rearranges to form γ-ketoaldehyde secoprostanoids. These compounds have been termed levuglandin E_2 and D_2 because of their structural similarities with levulinaldehyde. Levuglandins comprise approximately 20% of the total rearrangement products of PGH_2 (5).

Initially we reported the formation of F_2-IsoPs, which are produced by reduction of the IsoP endoperoxide intermediates (4). We recently discovered a key role for glutathione in effecting the reduction of IsoP endoperoxides to F_2-IsoPs (7). However, the reduction of IsoP endoperoxides is not completely efficient. In this regard, we have shown that the IsoP endoperoxides undergo rearrangement *in vivo* to form E_2-IsoPs, D_2-IsoPs, and isothromboxanes (8, 9).

Based on the observation that the IsoP endoperoxides undergo rearrangement *in vivo*, we explored whether such rearrangement also results in the formation of levuglandin-like compounds, for which we propose the term isolevuglandins (IsoLGs). Our interest in this possibility stems from the fact that the γ-ketoaldehyde moiety confers remarkable reactivity to these compounds. In this regard, Salomon and colleagues (10, 11) have shown that LGE_2 rapidly adducts to amines and, in addition, readily undergoes further reaction to form extensive protein-protein and protein-DNA cross-links. Thus, if formed, IsoLGs might participate as important mediators of oxidant injury. Therefore, we undertook studies to explore whether IsoLGs are formed during oxidation of arachidonic acid and studies to elucidate the nature of the IsoLG adduct

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[2] The abbreviations used are: 4-HNE, 4-hydroxynonenal; MDA, malondialdehyde; LDL, low density lipoprotein; apoB-100, apolipoprotein B; PG, prostaglandin; IsoPs, isoprostanes; LG, levuglandin; IsoLGs, isolevuglandins; GC, gas chromatography; MS, mass spectrometry; NICI, negative ion chemical ionization; EI, electron impact ionization; PBS, phosphate-buffered saline; LC, liquid chromatography; ESI, electrospray ionization; MS/MS, tandem mass spectrometry; SRM, selected reaction monitoring; TMS, trimethylsilyl; CID, collision-induced dissociation; BSA, bovine serum albumin; BSTFA, *N,O*-bis(trimethylsilyl)trifluoroacetamide; AAPH, 2,2′-azobis(2-amidinopropane)HCl; HPLC, high pressure liquid chromatography.

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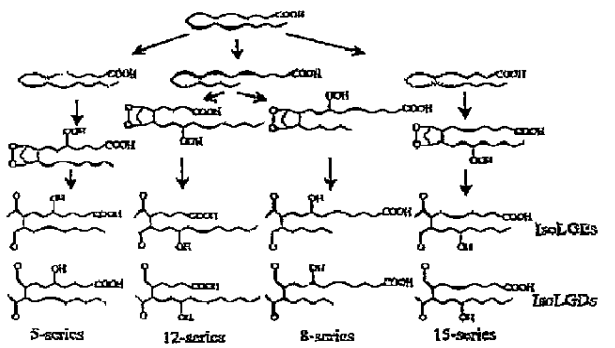

FIG. 1. **Predicted mechanism of formation of IsoLGs via the IsoP pathway.** Four IsoP bicyclic endoperoxide regioisomers are formed which then undergo rearrangement to form four E_2-IsoLG and four D_2-IsoLG regioisomers. Each E_2-IsoLG and D_2-IsoLG regioisomer is theoretically comprised of four racemic diastereomers.

that would be formed with lysine residues on proteins. The mechanism by which IsoLGs are generated by free radical-induced oxidation of arachidonic acid is shown in Fig. 1. Four regioisomers of both D_2-IsoLGs and E_2-IsoLGs are formed, each of which is theoretically comprised of four racemic diastereomers, for a total of 32 IsoLGE_2 and 32 IsoLGD_2 compounds. The designation "D" and "E" is the same as for the cyclooxygenase-derived LGs and refers to the location of the keto group. If the keto group is located at C-9, the molecule is designated an E_2-IsoLG, and if it is located at C-11, the molecule is designated as a D_2-IsoLG. Salomon and co-workers (12) had previously used a carbon numbering system from 1 to 17 beginning with the carboxyl carbon, and the chains containing the carbonyl groups were considered as substituents. However, to retain consistency with the official nomenclature for isoprostanes that has been approved by the Eicosanoid Nomenclature Committee, sanctioned by JCBN of IUPAC (13), the original 20 carbon numbering system used for the IsoP endoperoxide precursors will be retained. In accordance with the IsoP nomenclature, the different regioisomers are designated by the carbon number on which the side chain hydroxyl is located, as indicated in Fig. 1.

EXPERIMENTAL PROCEDURES

Oxidation of Arachidonic Acid—Five mg of arachidonic acid (Nu Chek Prep, Elysian, MN) or 1-palmitoyl, 2-arachidonoyl-glycero-3-phosphocholine (Sigma) was dissolved in 200 μl of ethanol and oxidized using a mixture of ferric chloride (1 mM), ADP (200 mM), and ascorbate (100 mM) in 5 ml of 50 mM phosphate buffer (pH 7.4) at 37 °C for 6 h.

Mass Spectrometric Analysis of IsoLGs—Following oxidation of arachidonic acid or 1-palmitoyl, 2-arachidonoyl-glycero-3-phosphocholine, compounds were converted to O-methyloxime derivatives by addition of 3% methoxyamine·HCl and incubated for 45 min at room temperature. Compounds formed from the oxidation of phosphatidylcholine were subsequently subjected to base hydrolysis with 7.5% KOH in 50% aqueous methanol for 30 min at 37 °C. Samples were then acidified to pH 3 with 1 N HCl and diluted. 1–4 ng of bis-[$^2H_3$]*O*-methyloxime-LGE_2, which was formed by treatment of synthetic LGE_2 (14) with [$^2H_3$]methoxyamine·HCl (Regis, Morton Grove, IL), was added as an internal standard. The concentration of the bis-[$^2H_3$]*O*-methyloxime-LGE_2 was standardized against $PGF_{2\alpha}$ by gas chromatography (GC)/negative ion chemical ionization (NICI)/mass spectrometry (MS). The sample was then applied to a C_{18} Sep-Pak column (Waters Associates, Milford, MA) that had been pre-conditioned with 5 ml of methanol and 10 ml of pH 3 water. The Sep-Pak was washed sequentially with 10 ml of pH 3 water and 10 ml of heptane/ethyl acetate (99:1 v/v), and the IsoLGs were then eluted with 10 ml of heptane/ethyl acetate (1:1 v/v). The heptane/ethyl acetate was dried under a stream of N_2. The samples were then converted to a pentafluorobenzyl ester derivative by treatment with 40 μl of 10% pentafluorobenzyl bromide in acetonitrile and 20 μl of 10% *N,N*-diisopropylethylamine in acetonitrile for 20 min at 37 °C. Samples were then subjected to thin layer chromatography (TLC) using silica gel plates (VWR Scientific, Atlanta, GA) using the solvent heptane/ethyl acetate (60:40 v/v). Compounds migrating 2 cm above and 0.5 cm below the bis-*O*-methyloxime, pentafluorobenzyl ester derivative of LGE_2 were scraped and extracted from the silica gel with ethyl acetate. IsoLGs were then converted to trimethylsilyl (TMS) ether derivatives by treatment with 10 μl of dimethylformamide and 10 μl of *N,O*-bis(trimethylsilyl)trifluoroacetamide (BSTFA) (Supelco, Bellefonte, PA) and analyzed by GC/NICI/MS. GC was performed with a 12-m DB 1701 fused silica capillary column (J & W Scientific, Folsom, CA) heated from 190 to 300 °C at 20 °C/min with a 0.2-min hold. Source temperature was 250 °C.

Determination of functional groups was accomplished by subjecting 2 ml of an arachidonate oxidation mixture to derivatization with methoxyamine·HCl or [$^2H_3$]methoxyamine·HCl. No internal standard was added. Derivatization was continued as above except each sample was divided equally after TLC and half derivatized with BSTFA as above and half with [$^2H_9$]BSTFA (Deutero-Regisil-d^{18}, Regis, Morton Grove, IL) (6 μl + 6 μl dimethylformamide). Samples were then analyzed by GC/NICI/MS, employing selected ion monitoring for the [M − $^{\cdot}CH_2C_6F_5$]$^-$ ion of each derivative. Disappearance of signals was monitored as well as appearance of signals, *i.e.* the presence of two carbonyl groups was indicated by the disappearance of signals at *m/z* 481 and the appearance of signals at *m/z* 487 in samples treated with [$^2H_3$]methoxyamine·HCl and BSTFA, and the presence of a single hydroxyl group was indicated by the disappearance of signals at *m/z* 481 and the appearance of signals at *m/z* 490 in samples treated with methoxyamine·HCl and [$^2H_9$]BSTFA.

For analysis of IsoLGs by electron impact ionization (EI)/MS, methoximated IsoLGs were purified by reverse phase HPLC on an Econosil C_{18} column (Alltech Associates, Deerfield, IL) utilizing a solvent system of 45% acetonitrile in water with 0.1% acetic acid. One-ml fractions were collected. Fractions containing IsoLGs were identified by analysis of aliquots as above by GC/NICI/MS. IsoLGs were detected eluting over approximately 30 fractions. Eight fractions eluting at approximately 30 ml, which contained a high concentration of IsoLGs, were pooled. This purification procedure was repeated several times to obtain sufficient material for analysis by GC/EI/MS. GC/EI/MS analysis yielded a single prominent chromatographic peak with a smaller shoulder that contained ions consistent with mass spectra of IsoLGs at an approximate retention time of 9 min. This retention time is longer than the retention time of IsoLGs in the GC/NICI/MS experiments described above (approximately 6 min) because a longer GC column was used to achieve more effective separation of IsoLGs from other potentially interfering compounds. Spectra were displayed by averaging the scans across the chromatographic peaks. The source temperature was 200 °C, the ionization energy 70 eV, and the transfer line and injector each held at 250 °C.

Mass Spectrometric Analysis of F_2-IsoPs and D_2/E_2-IsoPs—D-ring, E-ring, and F-ring IsoPs generated from oxidation of arachidonic acid were quantified by GC/NICI/MS as described (4, 8).

Comparison of the Rate of Adduction of LGE_2 and 4-HNE to Albumin—0.1 mM LGE_2 and 0.1 mM 4-HNE were incubated with bovine serum albumin (BSA) (5 ml at 20 mg/ml in Hanks' balanced salt solution at 37 °C). 100-μl aliquots were removed at indicated time points, and free unadducted LGE_2 was quantified by GC/NICI/MS as described above. Free unadducted 4-HNE was measured by colorimetric assay (Oxis International, Portland, OR).

Formation and Analysis of LGE_2-Lysine Adducts—LGE_2 (1 mM) was incubated with [^{3}H]lysine (1 mM; 17,000 cpm/mg) in 1 ml of phosphate-buffered saline at 37 °C for 4 h. Incubations were also performed in which lysine was replaced with [U-^{13}C]lysine (Cambridge Isotope Laboratories, Andover, MA) or N^{α}-acetyl-lysine methyl ester (Sigma). 100-μl aliquots of the incubation mixtures were analyzed by liquid chromatography (LC)/electrospray ionization (ESI)/MS in the positive ion mode using a Waters 2.1 × 150 mm C_{18} column and a water/acetonitrile gradient (3%/min; hold 5 min) at 0.2 ml/min. Auxiliary gas pressure was 70 pounds/square inch; sheath gas pressure was 10 pounds/square inch. The voltage on the capillary was 20.5 V, the capillary temperature 200 °C, and the tube lens voltage 90 V. Parent ions were scanned from *m/z* 400 to *m/z* 500. Collision-induced dissociation (CID) of molecular ions of the putative lactam and hydroxylactam adducts formed in these incubations was performed from −10 to −40 eV, scanning daughter ions from *m/z* 50 to *m/z* 500 (up to *m/z* 560 for the N^{α}-acetyl-lysine methyl ester derivative). Spectra shown were obtained at −28 eV. CID gas was argon with a pressure set at 2.0 millitorr. Spectra were displayed by averaging scans across the chromatographic peaks.

Formation and Analysis of IsoLG Lysine Adducts—100 mg of arachi-

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donic acid was oxidized as described above in the presence of 100 mg of lysine. The incubation mixture was then loaded onto a C_{18} Sep-Pak cartridge in pH 3 water, washed sequentially with 10 ml of water, 10 ml of heptane, and 10 ml of heptane/ethyl acetate (1:1 v/v). Adducts were eluted with 10 ml methanol/ethyl acetate (35:65 v/v). The organic solvents were then dried under a stream of N_2, and an aliquot was analyzed by LC/ESI/MS/MS using conditions described above except the LC flow rate was 0.1 ml/min. MS/MS analysis was carried out using selected reaction monitoring (SRM) of daughter ions produced from CID of $[MH]^+$ at −28 eV. 1/20 of this preparation was analyzed by SRM of daughter ions of the lactam adducts, and approximately 1/3 was analyzed by SRM of daughter ions of the hydroxylactam adducts.

Isolation and Analysis of IsoLG Adducts on Oxidized LDL—LDL was isolated from 10 ml of plasma obtained from normal human volunteers using a low temperature ethanol precipitation procedure (15). Briefly, the plasma was stirred with a 25% ethanol solution at −5 °C for 15 min and then centrifuged at 3000 × *g* for 30 min at −5 °C. The precipitated LDL was then suspended in 50 mM potassium phosphate buffer (pH 7.4) at a concentration of 10 mg of protein/ml and oxidized with 0.1 mM 2,2′-azobis(2-amidinopropane) HCl (AAPH) (Polysciences, Warrington, PA) for 4 h. The LDL was subsequently re-precipitated as above and delipidated (15). Briefly, the LDL was diluted to 10 mg of protein/ml and then sequentially extracted with ethanol/diethyl ether (2:3 v/v) for 24 h; ethanol/ether (3:1 v/v) for 24 h; ethanol/ether (1:1 v/v) for 30 min; ethanol/ether (1:3 v/v) for 30 min; and finally briefly washed with ether. The temperature was maintained at −15 °C during the delipidation procedure. The protein was recovered after each extraction by centrifugation at 850 × *g* for 20 min. The protein was re-dissolved by suspending it in ether, and 0.2 N NaOH was then added. The mixture was gently bubbled with N_2 until the ether evaporated and then incubated for 2 h at room temperature to hydrolyze IsoLG protein adducts that may have formed with IsoLG esterified to LDL lipids. The pH was then adjusted to 7.5, and the protein was subjected to complete enzymatic digestion to individual amino acids as described (16). Briefly, the protein concentration was adjusted to 1 mg/ml. 0.1 volume of a 1 mg/ml solution of Pronase (Calbiochem) was added, and the mixture was incubated at 37 °C for 18 h. The pH was then adjusted to 8.5, and $MgCl_2$ was added to a final concentration of 4.8 mM. 0.1 volume of leucine aminopeptidase (Sigma) activation solution was subsequently added. Leucine aminopeptidase was activated by incubating 225 μg/ml in 10 mM Tris-HCl buffer (pH 8.5) with 1 mM $MnCl_2$ for 2–3 h at 40 °C and subsequently added to the digest, which was then incubated at 37 °C for 18 h at room temperature. The compounds were then extracted using a C_{18} Sep-Pak cartridge as described above. The eluate was dried under a stream of N_2, and IsoLG/lysine adducts were analyzed by LC/ESI/MS/MS as described for the IsoLG adducts above. Before analysis, a mixture of $[^{13}C_6]$lactam and $[^{13}C_6]$hydroxylactam IsoLG adducts was added to allow quantification of the amount of IsoLG adducts detected. These compounds were formed by oxidation of 25 mg of arachidonic acid in the presence of [^{3}H]lysine (50 × 10^9 cpm; 10,000 cpm/μg) (NEN Life Science Products) diluted with 1 mg of $[^{12}C_6]$lysine (Cambridge Isotope Laboratories, Andover, MA). The [^{3}H]- and $[^{13}C_6]$lysine-IsoLG lactam, and hydroxylactam adducts formed were then purified by extraction on C_{18} Sep-Pak cartridges as above and HPLC (water to acetonitrile in 30 min with 5-min water pre-wash). HPLC fractions containing the lysyl IsoLG adducts were detected by LC/ESI/MS analysis, and the concentration was determined by the specific activity of the [^{3}H]lysine.

RESULTS

Identification of IsoLGs Formed during Oxidation of Arachidonic Acid in Vitro—GC/NICI/MS analysis of oxidized arachidonate mixtures revealed a series of compounds with characteristics of IsoLGs (Fig. 2). The lower *m/z* 487 chromatogram displays a series of incompletely resolved peaks corresponding to the $[M - {}^{\cdot}CH_2C_6F_5]^-$ ion of the bis-$[^2H_3]$*O*-methyloxime LGE_2 internal standard. These represent the four methoxime isomers (two *syn* and two *anti*) resulting from methoximation of the two carbonyl groups of the internal standard. At a similar retention time in the upper *m/z* 481 chromatogram are a series of peaks with the $[M - {}^{\cdot}CH_2C_6F_5]^-$ ion of IsoLGs. The pattern of the putative IsoLG compounds differs from that seen in the *m/z* 487 chromatogram, consistent with the formation of multiple $IsoLGE_2$ and $IsoLGD_2$ isomers (see Fig. 1). Virtually identical results were obtained from the analysis of IsoLGs formed during oxidation of 1-palmitoyl, 2-arachidonoyl-3-glycero-phosphocholine (data not shown).

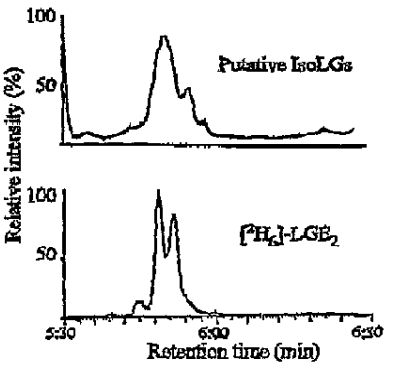

FIG. 2. **Selected ion current chromatograms obtained from GC/NICI/MS analysis for IsoLGs formed during oxidation of arachidonic acid *in vitro*.** Compounds were analyzed as a pentafluorobenzyl ester, *O*-methyloxime, trimethylsilyl ether derivative monitoring the $[M - {}^{\cdot}CH_2C_6F_5]^-$ ions at *m/z* 481 for IsoLGs and *m/z* 487 for the deuterated internal standard, the bis-$[^2H_3]$*O*-methyloxime derivative of synthetic LGE_2.

Additional studies were then undertaken to confirm the identity of these compounds as IsoLGs. When analyzed as a $[^2H_3]$*O*-methoxylamine derivative, all of the *m/z* 481 peaks shifted upwards 6 Da to *m/z* 487, indicating the presence of two carbonyl groups. When analyzed as a $[^2H_9]$TMS ether derivative, all of the *m/z* 481 peaks shifted upward 9 Da to *m/z* 490, indicating the presence of one hydroxyl group (data not shown). These compounds were then analyzed by GC/EI/MS after partial purification by HPLC. Mass spectra were obtained that were consistent with the formation of both E_2- and D_2-IsoLGs. One of the mass spectra obtained is shown in Fig. 3*A*. Although it is unlikely that this is a mass spectrum of a single IsoLG isomer because the preparation analyzed was only partially purified, this mass spectrum is consistent with a major component being a 15-series $IsoLGD_2$ compound. The spectrum is characterized by an intense molecular ion at *m/z* 662 and intense ions at *m/z* 631 (M − 31), loss of ${}^{\cdot}OCH_3$ from a methoxime group, *m/z* 591 (M − 71), loss of ${}^{\cdot}CH_2(CH_2)_3CH_3$ from fragmentation between C-15 and C-16 on the lower side chain, *m/z* 559 (M − 71 − 32), the loss of ${}^{\cdot}CH_2(CH_2)_3CH_3 + HOCH_3$, *m/z* 541(M − 90 − 31), loss of $Me_3SiOH + {}^{\cdot}OCH_3$, *m/z* 501 (M − 90 − 71), loss of $Me_3SiOH + {}^{\cdot}CH_2(CH_2)_8CH_3$, *m/z* 469 (M − 173), loss of ${}^{\cdot}CH_2(OSiMe_3)(CH_2)_4CH_3$ from fragmentation between C-14 and C-15 on the lower side chain. A mass spectrum of authentic LGE_2 is shown in Fig. 3*B*. Notable are the striking similarities in the high mass ions present and their relative abundances in the spectra in Fig. 3, *A* and *B*. The presence of ions involving the loss of 71 and 173 Da in both mass spectra resulting from fragmentation adjacent to the carbon on the lower side chain on which the TMS ether group is attached supports the identity of a major component of the mass spectrum in Fig. 3*A* as a 15-series IsoLG. An ion at *m/z* 418 is present in both the IsoLG and LGE_2 spectra, but its origin is unclear; it may derive from a minor common decomposition product. Intense fragmentation ions are present in the mass spectrum of authentic LGE_2 resulting from fragmentation between C-8 and C-12, one representing the upper portion of the molecule (*m/z* 392) and one representing the lower portion of the molecule (*m/z* 270). These ions are also observed in the IsoLG spectrum, indicating that it may also contain a 15-series $IsoLGE_2$ compound as a minor component. The base ion in the mass spectrum in Fig. 3*A* is *m/z* 284. This origin of this ion would be consistent with the lower portion of a 15-series $IsoLGD_2$ molecule, as a result of fragmentation between

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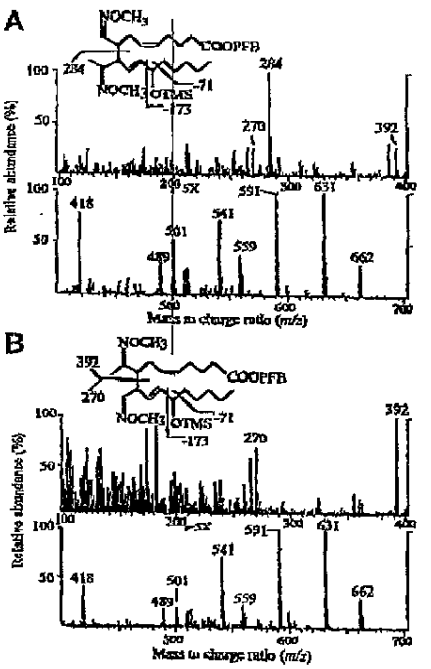

FIG. 3. **Electron ionization mass spectra obtained from the analysis of partially purified IsoLGs (*A*) and of synthetic LGE_2 (*B*) as pentafluorobenzyl ester, bis-*O*-methyloxime, trimethylsilyl ether derivatives.** Interpretations of ions are discussed in detail in the text.

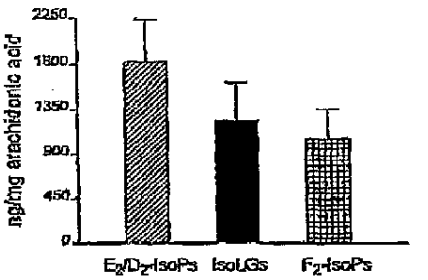

FIG. 4. **Comparison of the relative amounts of IsoLGs, F_2-IsoPs, and E_2/D_2-IsoPs formed following oxidation of arachidonic acid *in vitro*.** Five mg of arachidonic acid was oxidized for 4 h with iron/ADP/ascorbate.

C-8 and C-12. However, unlike the mass spectrum of authentic LGE_2, an ion representing the upper portion of the molecule is not present. Possible reasons for this include different relative abundances of these two ions in different IsoLG isomers or in LGD *versus* LGE compounds. The latter possibility cannot be explored because authentic LGD_2 is not available. Salomon and colleagues have attempted the synthesis of LGD_2 but found it to be highly unstable under the conditions used for its synthesis (12).

Relative Amounts of IsoLGs and IsoPs Produced during Oxidation of Arachidonic Acid in Vitro—To evaluate the quantitative relevance of the formation of IsoLGs, we compared the amounts of IsoLGs with the amounts of F_2-IsoPs and E_2/D_2-IsoPs formed during oxidation of arachidonic acid *in vitro*. Surprisingly, we found that the amounts of IsoLGs formed were approximately equivalent with the amounts of F_2-IsoPs and were only slightly less than the amounts of E_2/D_2-IsoPs (Fig. 4). These data suggest strongly that IsoLGs can be produced as products of the IsoP pathway at levels that can potentially have significant biological impact.

LGs Readily Form Covalent Adducts With Proteins—Having established that IsoLGs can be formed in significant amounts during oxidation of arachidonic acid *in vitro*, we explored whether we could detect their formation in a number of biological systems *in vitro* and *in vivo*. Despite the fact that readily detectable levels of F_2-IsoPs are present in less than 1 ml of normal human plasma and urine, we could not detect IsoLGs in 3 ml of either human plasma or urine. Furthermore, we could not detect the formation of IsoLGs *in vivo* in 1 g of liver from rats treated with CCl_4 to induce an intense oxidant injury to the liver which was associated with a marked increase in the formation of F_2-IsoPs. We also could not detect the formation of IsoLGs *in vitro* following (*a*) iron/ADP/ascorbate-induced oxidation of liver microsomes (17) prepared from 5 g of rat liver or (*b*) following copper-mediated oxidation of LDL (18) isolated from 2.5 ml of plasma, both of which again were accompanied by marked increases in F_2-IsoP formation. The overriding difference between these experiments and those in which we oxidized arachidonic acid *in vitro* is the presence of protein. We hypothesized that the failure to detect IsoLGs in these biological systems may be attributed to extremely rapid adduction of IsoLGs to proteins. Thus, we attempted to intercept the formation of IsoLG adducts in the microsome and LDL oxidation mixtures by adding oxime reagents to a final concentration of 3% to derivatize the carbonyls, which would prevent IsoLG adduction to amines. Although this approach effectively converted the carbonyls of E_2/D_2-IsoPs that were formed in these experiments to oxime derivatives, it did not successfully trap free IsoLGs. This suggested either that IsoLGs were not formed or that under the experimental conditions used, their reaction with proteins was much faster than their reaction with the oxime reagents.

In an attempt to gain support for our hypothesis that IsoLGs could not be detected as free dicarbonyl compounds in biological systems because they adduct to proteins with extreme rapidity, we compared the rate of adduction of LGE_2 and 4-HNE with BSA, as a model protein. We thought a comparison of the rate of adduction of LGE_2 with that of 4-HNE would be informative because 4-HNE, although considered to be one of the most highly reactive products of lipid peroxidation yet identified, can be detected in free form in biological systems (2). The rate of adduction of these two compounds was assessed by monitoring the decline in levels of free compounds measured in aliquots removed at various times during incubations consisting of LGE_2 or 4-HNE with a 3 molar excess of BSA. As shown in Fig. 5, levels of free LGE_2 dropped precipitously during the initial 60 s of the incubation; notably more than 50% had adducted within 20 s. Of note, free LGE_2 did not decline completely to undetectable levels but leveled off between 5 and 10% of the amount detected at time 0. This can likely be attributed to facile migration of the Δ^{12} double bond to the Δ^{12} position, which may decrease the reactivity of the molecule (12). A small amount of Δ^{12}–LGE_2 may have been present in the synthetic preparation, or migration of the double bond may have occurred during the incubation since the double bond migration has been shown to be catalyzed by buffer ions (12). In striking contrast to the remarkably fast rate of adduction of LGE_2 to BSA that was observed, ~50% of 4-HNE had still not adducted after 1 h. The rate of adduction of 4-HNE to albumin found in this study agrees closely with that reported previously by Curzio *et al.* (19). These data indicated that the rate at which LGE_2 adducts to proteins exceeds that of 4-HNE by several orders of magnitude and provided a very plausible explanation for our

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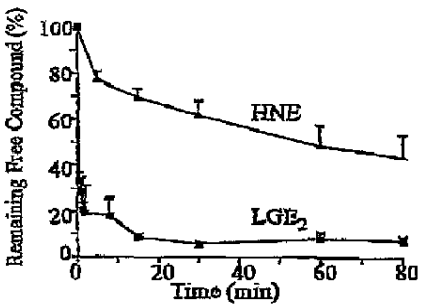

FIG. 5. **Comparison of the rates of covalent adduction of LGE_2 and 4-HNE to BSA.** The formation of covalent adducts was assessed by monitoring the disappearance of free compounds over time and expressed as a percent of the amount of free compound present at time 0. The proposed explanation for why the levels of LGE_2 do not continue to fall to undetectable levels but plateau between 5 and 10% of the amount of LGE_2 present at time 0 is discussed in the text.

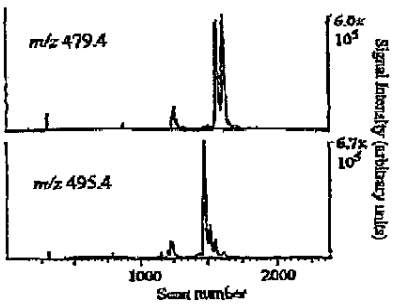

FIG. 6. **Selected ion current chromatograms of $[MH]^+$ ions *m/z* 479 and *m/z* 495 from an LC/ESI/MS analysis of adducts formed following incubation of LGE_2 with lysine.**

inability to detect IsoLGs in free form in biological systems containing protein.

Identification of LGE_2-Lysine Adducts—We then sought to structurally identify the nature of LG adducts using LC/ESI/MS/MS. Initial studies were carried out using synthetic LGE_2 as a model for IsoLGs. Based on previous data reported by Salomon and colleagues (20), we predicted that a major protein adduct would be a pyrrole produced from reaction of LGE_2 with the ε-amino group of lysyl residues. In initial studies, we used free lysine to model this reaction which seemed valid because the α-amino group of free lysine is only about 1/6 as reactive as the ε-amino group (16). The predicted $[MH]^+$ ion for the LGE_2-lysine pyrrole adduct is *m/z* 463. Full scanning spectrum analysis, however, consistently revealed the presence of intense ions at *m/z* 479 and 495, rather than an ion at *m/z* 463. Selected ion current chromatograms of *m/z* 479 and *m/z* 495 obtained from analysis of an incubation of LGE_2 with lysine is shown in Fig. 6. Notably, these ions are 16 and 32 Da higher than the $[MH]^+$ ion of the pyrrole adduct. Relevant to this finding were previous data demonstrating that pyrroles can undergo autoxidation to form lactams and hydroxylactams, which have masses 16 and 32 Da higher, respectively, than the corresponding pyrroles (21). The mechanism by which lactams and hydroxylactams are predicted to form by autoxidation of pyrroles is shown in Fig. 7. The LGE_2 pyrrole would be expected to be highly susceptible to autoxidation because it is highly alkylated and electron-rich (20). Consistent with such a prediction, Salomon and colleagues (20) found that the pyrrole formed by LGE_2 reaction with neopentylamine was highly unstable and could not be isolated unless air was rigorously excluded. We therefore attempted to prevent oxidation of the LGE_2-lysine pyrrole that was predicted to have been formed by incubating LGE_2 with lysine under argon in deaerated buffer. Under these conditions, we did see a new peak at *m/z* 463 that eluted at approximately the same retention volume as the lactam and hydroxylactam adducts, consistent with an LGE_2-lysine pyrrole. However, the CID mass spectrum of this compound only produced ions at *m/z* 445, formed by the loss of H_2O, and *m/z* 363, formed by the loss of 100 Da. The loss of 100 Da is speculated to occur from fission of the bond between C-14 and C-15 with transfer of a proton as described by Murphy and colleagues (22) during CID of prostaglandins. Although suggestive, it could not be concluded with certainty that this compound was in fact the lysyl-LGE_2 pyrrole because of the limited structural information provided by the CID mass spectrum.

The putative lysyl-LGE_2 lactam and hydroxylactam adducts were then structurally characterized further by CID of the

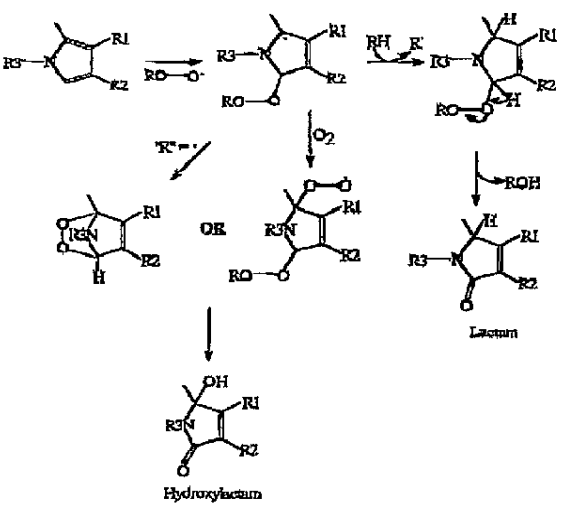

FIG. 7. **Proposed mechanism of formation of lactams and hydroxylactams by autoxidation of pyrroles (adapted from Ref. 21).**

$[MH]^+$ ions, *m/z* 479 and *m/z* 495, respectively. CID of the putative lactam adduct produced informative daughter ions at *m/z* 461, *m/z* 415, *m/z* 346, *m/z* 332, and *m/z* 84 (Fig. 8*A*). CID of the putative hydroxylactam adduct produced analogous daughter ions at *m/z* 477, *m/z* 459, *m/z* 413, *m/z* 330, and *m/z* 84 (Fig. 8*B*). Insight into the structures of these fragment ions was obtained by analysis of the LGE_2 adducts formed with [$^{13}C_6$]lysine and N^{α}-acetyl-lysine methyl ester. The proposed structures for these ions and the corresponding ions in the CID spectra of the adducts formed with the lysine analogs are shown in Figs. 9 and 10. The ion shifts in the CID spectra of the adducts formed with the lysine analogs support the proposed structures of these ions. However, the precise mechanism of their formation remains speculative, except for ions at *m/z* 461 in the lactam CID spectrum and *m/z* 477 and *m/z* 459 in the hydroxylactam CID spectrum, representing the loss of H_2O (*m/z* 461 and *m/z* 477) and 2× H_2O (*m/z* 459).

It is important to mention that we cannot be quantitatively precise about the yields of the lactam and hydroxylactam adducts for reasons related to the labile and highly reactive nature of LGE_2. First, the synthetic LGE_2 is impure, and thus the weight of the preparation is an unreliable indicator of the actual amount of LGE_2 present. The LGE_2 cannot be purified from precursors and side products from the final reaction in the synthesis because it degrades extensively during column chro-

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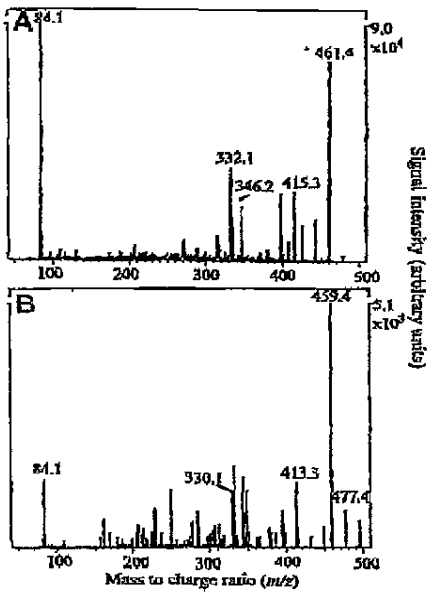

FIG. 8. **LC/ESI/MS/MS analysis of LGE_2-lysine adducts.** The $[MH]^+$ ions of the putative lactam (*A*) and hydroxylactam (*B*) adducts, *m/z* 479 and *m/z* 495, respectively, were subjected to CID at −28 eV and daughter ions scanned from *m/z* 50 to *m/z* 500. Spectra were obtained by averaging scans across the peaks observed. The interpretations of the structures of individual ions are detailed in Figs. 9 and 10.

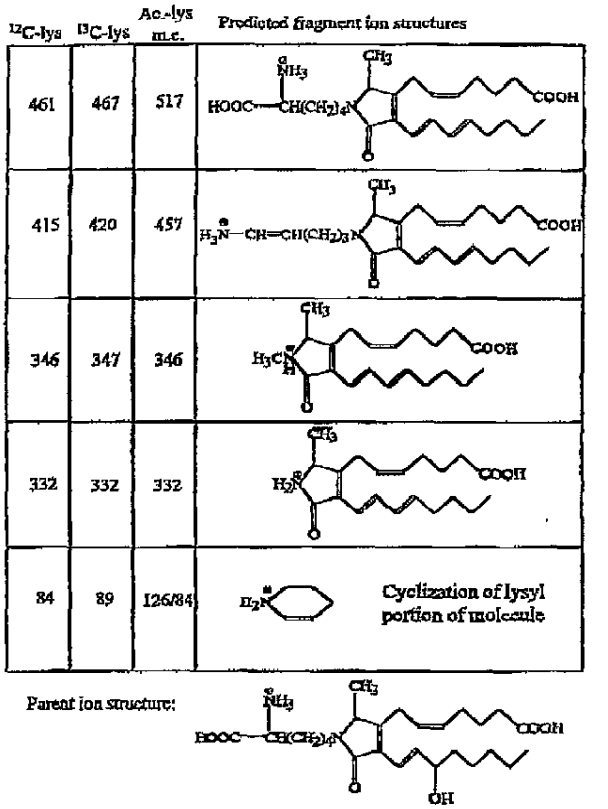

FIG. 9. **Interpretations of the structures of individual CID daughter ions of the lysyl LGE_2 lactam adducts.** LGE_2 was reacted with free lysine (*column 1*), [$^{13}C_6$]lysine (*column 2*), or N^{α}-acetyl-lysine methyl ester (*column 3*), producing the corresponding lactam adduct $[MH]^+$ ions at *m/z* 479, *m/z* 485, and *m/z* 535, respectively. These ions were then subjected to LC/ESI/MS/MS, yielding corresponding daughter ions noted that supported the interpretations of the ion structures shown on the *right*.

matography (12). We also cannot accurately quantify the amount of LGE_2 present by selected ion monitoring GC/NICI/MS comparing the amount present to a known amount of deuterated internal standard. This is because the double bond at the Δ^{13} position, as mentioned above, can readily migrate to the Δ^{12} position, which changes the reactivity of the molecule (12). GC/NICI/MS analysis cannot distinguish between the reactive Δ^{13}-LGE_2 and the relatively unreactive Δ^{12}-LGE_2. In this regard, we have found that the amount of LGE_2 that adducts decreases significantly over time during storage, even though the amount of signal seen by GC/NICI/MS analysis remains essentially constant. This phenomenon is presumably due to conversion of synthetic Δ^{13}-LGE_2 to Δ^{12}-LGE_2 during storage. Thus, it is not possible in any given experiment to know precisely how much reactive Δ^{12}-LGE_2 is added to the incubation. As discussed previously, this conversion may also account for the apparent biphasic rate of adduction of LGE_2 to BSA seen in Fig. 5. The other issue that confounds quantification is related to the remarkable proclivity of LGE_2 to induce cross-links (10, 11). In this regard, HPLC analysis of LGE_2 that has been incubated with a molar excess of tritiated lysine results in a large amount of radioactivity that chromatographs as multiple unresolved peaks eluting over many fractions. This broad slur of tritiated reaction products elutes at a retention volume that is widely separated from free lysine and elutes over a much broader range than the lactam and hydroxylactam adducts. Although this material is not amenable to analysis by standard approaches utilized in these studies, we speculate that this material represents cross-linked species. Thus lactam and hydroxylactam adducts do not account for all or even most of the adducts formed during LGE_2 reaction with proteins.

Identification of IsoLG-Lysine Adducts—We then utilized the information obtained from analysis of LGE_2-lysine adducts to characterize the formation of IsoLG-lysine adducts. Arachidonic acid was oxidized in the presence of lysine, and adducts formed were analyzed by LC/ESI/MS. The analysis revealed compounds at *m/z* 479 and *m/z* 495 that had LC elution volumes consistent with the formation of IsoLG-lysine lactam and hydroxylactam adducts, respectively. LC/ESI/MS/MS analysis revealed that these compounds formed the same daughter ions that had been observed for LGE_2-lysine lactam and hydroxylactam adducts (Fig. 11). No peaks at *m/z* 463, indicative of pyrrole adducts, were detected. Experiments excluding air in an attempt to obtain evidence for the formation of pyrrole adducts could not be performed because of the requirement of oxygen during oxidation of arachidonic acid. Notably, the chromatograms obtained from analysis of IsoLG adducts reveal multiple *m/z* 479 and *m/z* 495 peaks, consistent with the formation of a series of IsoLG isomers.

Detection of IsoLG/Apolipoprotein Adducts in Oxidized LDL—We then sought to determine if we could detect IsoLG/apoB-100 adducts following oxidation of LDL. We considered these experiments to be very informative since, as mentioned previously, our attempts to detect unadducted free IsoLGs during oxidation of LDL were not successful. For these analyses, as outlined under "Experimental Procedures," LDL was oxidized for 4 h with AAPH and delipidated, and the apoB-100 protein was then digested to individual amino acids by sequential treatment with Pronase and leucine aminopeptidase (16). Na-

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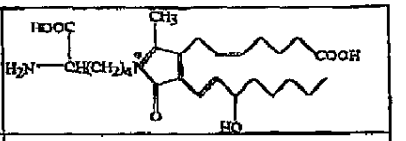
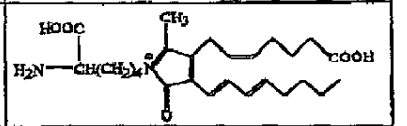
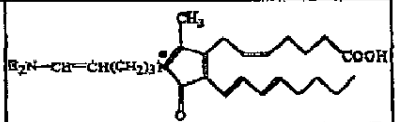
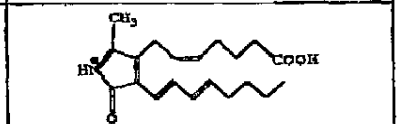
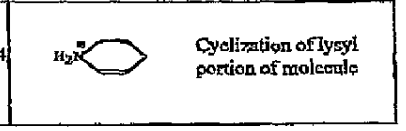
^{12}C-lys	^{13}C-lys	Ac.-lys m.e.	Predicted fragment ion structures
477	483	533	
459	465	515	
413	418	455	
330	330	330	
84	89	126/84	 Cyclization of lysyl portion of molecule

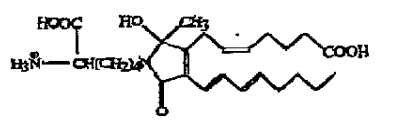
Parent ion structure: 

FIG. 10. **Interpretations of the structures of individual CID daughter ions of the hydroxylactam adducts.** LGE_2 was reacted with free lysine (*column 1*), [$^{13}C_6$]lysine (*column 2*), or N^{α}-acetyl-lysine methyl ester (*column 3*), producing the corresponding hydroxylactam [MH]$^+$ ions at *m/z* 495, *m/z* 501, and *m/z* 551, respectively. These ions were then subjected to LC/ESI/MS/MS, yielding corresponding daughter ions noted that supported the interpretations of the ion structures shown on the *right*.

tive LDL (not subjected to oxidation) was also treated in an identical fashion. The amino acid mixture was then analyzed by LC/ESI/MS/MS, employing SRM. No adducts were detected in native LDL. However, as shown in Fig. 12, intense signals consistent with the presence of IsoLG lactam and hydroxylactam adducts were detected in the hydrolysate prepared from oxidized LDL. These compounds had molecular ions ([MH]$^+$) of *m/z* 479 and *m/z* 495 that transitioned to *m/z* 84.1 during CID, supporting their characterization as lactam and hydroxylactam adducts, respectively. These compounds also had the same LC elution volume as the internal standards, [$^{13}C_6$]lysine-IsoLG lactam and hydroxylactam adducts, at *m/z* 485 and *m/z* 501 that transitioned to *m/z* 89, respectively. Multiple peaks representing the [$^{13}C_6$]lysine internal standard adducts are present because these were generated by oxidation of arachidonic acid in the presence of [^{3}H]- and [$^{13}C_6$]lysine and thus consist of multiple IsoLG-lysine adducts. Approximately 1 nmol of IsoLG adduct was formed per 500 nmol of apoB-100 (500 ng/250 mg starting lipoprotein) as calculated by comparing the ratio of intensity of signals for adducts detected in oxidized LDL to signals for the known amount of ^{3}H- and $^{13}C_6$-adducts added.

DISCUSSION

These studies have identified a novel class of extremely reactive molecules that are generated as products of the IsoP

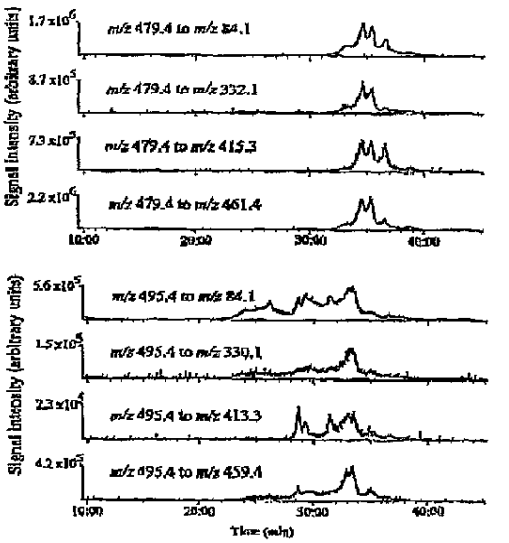


FIG. 11. **LC/ESI/MS/MS analysis of IsoLG-lysine adducts formed during oxidation of arachidonic acid, to generate IsoLGs, in the presence of lysine.** SRM monitoring of the transitions of *m/z* 479 (lactam adducts) and *m/z* 495 (hydroxylactam adducts) to the daughter ions indicated in the figure was performed. Interpretations of daughter ion structures are shown in Figs. 9 and 10.

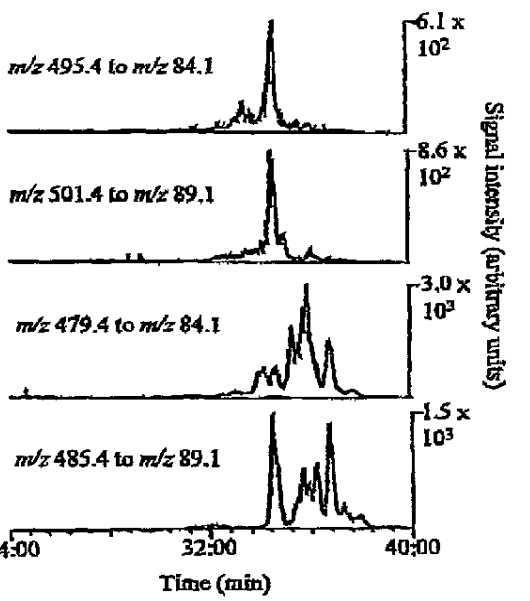


FIG. 12. **LC/ESI/MS/MS analysis of IsoLG-apoB-100 adducts from oxidized LDL.** LDL was subjected to oxidation, delipidated, and the apo-B-100 protein, then subjected to complete enzymatic hydrolysis to individual amino acids as described under "Experimental Procedures." Internal standards of [$^{13}C_6$]lactam and -hydroxylactam adducts, formed by oxidation of arachidonate in the presence of [$^{13}C_6$]lysine, were then added. The hydrolysate was analyzed by SRM of the following transitions: *m/z* 495.4 to *m/z* 84.1 (IsoLG-lysine hydroxylactam adducts from oxidized LDL); *m/z* 501.4 to *m/z* 89.1 (internal standard IsoLG [$^{13}C_6$]lysine hydroxylactam adducts); *m/z* 479.4 to *m/z* 84.1 (IsoLG-lysine lactam adducts from oxidized LDL); *m/z* 485.4 to *m/z* 89.1 (internal standard IsoLG [$^{13}C_6$]lysine lactam adducts).

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pathway. The relevance of the formation of these compounds relates to their remarkable proclivity to form covalent adducts with proteins and induce protein-protein and DNA-protein cross-links. Thus, these compounds might be expected to be highly injurious as a result of covalent modification of key biomolecules that are critical to normal cellular function and replication. Reactive aldehydes generated from lipid peroxidation, such as 4-HNE and MDA, form covalent adducts and are thought to be important mediators of some of the adverse sequelae of oxidant injury, including conversion of LDL to an atherogenic form (2, 3). The finding that IsoLG adducts could be easily detected in apoB protein following oxidation of LDL *in vitro* suggests that IsoLGs may also contribute to this pathogenetic process. Further studies quantitatively assessing the relative abundance of 4-HNE, MDA, and IsoLG adducts on apoB following oxidation of LDL should provide important insight into the relative extent to which these reactive molecules participate in the conversion of LDL to an atherogenic form. In addition, 4-HNE is considered to be one of the most reactive aldehydes generated as a product of lipid peroxidation (2). In this regard, the data obtained that demonstrated that LGE_2 adducts to BSA at a rate that exceeds that of 4-HNE by several orders of magnitude was a finding that dramatically highlights the highly reactive nature and potential importance of the generation of IsoLGs.

These studies open up numerous new avenues for potentially important scientific inquiry regarding the formation of IsoLGs in settings of oxidant injury. In addition to their ability to adduct to proteins, IsoLGs should also readily adduct to DNA. In this regard, Salomon and colleagues (11) have shown that LGE_2 can form DNA-protein cross-links in cultured cells. It is well recognized that free radical-induced DNA damage can lead to malignant transformation (23). However, nothing is known at present about the consequences of the formation of IsoLG-DNA adducts, *e.g.* what type(s) of mutation results from the formation of such adducts, whether these adducts are repaired, and if they are repaired, the rate at which this occurs.

The chemistry of the IsoLG reaction with amines is similar to other γ-dicarbonyl compounds. Thus, information regarding the pharmacological toxicology of γ-dicarbonyl compounds can provide a basis for hypotheses regarding the potential biological ramifications of the formation of IsoLGs. For example, the toxicity of one such compound that has been extensively studied is the hexane metabolite, 2,5-hexanedione. 2,5-Hexanedione has been shown to cause axonal degeneration through formation of pyrrole adducts and subsequent cross-linking of neurofilaments (24). This suggests, therefore, that similar effects may also result from the formation of IsoLGs during oxidative neuronal injury. In this regard, we recently described (25) the formation of IsoP-like compounds, termed neuroprostanes, *in vivo* from free radical induced oxidation of docosahexaenoic acid (C-22:6ωT3), which is highly enriched in neurons in the brain. In light of this finding, it is reasonable to speculate that reactive IsoLG-like compounds are also generated as products of the neuroprostane pathway, which in turn may induce neuronal injury. Potentially highly relevant in this regard is our recent discovery that levels of both neuroprostanes and IsoPs are significantly increased in cerebrospinal fluid from patients with Alzheimer's disease compared with age-matched control subjects (25, 26).

In summary, these studies have elucidated a novel class of extremely reactive compounds, IsoLGs, that are formed as products of the IsoP pathway. LGs exhibit a proclivity to adduct to proteins that far exceeds that of any other known reactive product of lipid peroxidation. The structural characterization of the nature of the lysyl IsoLG adducts formed on proteins reported herein provides key information that will allow us to explore the formation of IsoLGs *in vivo*. This should afford ample new avenues for investigation to elucidate the biological consequences of the formation of IsoLGs in settings of oxidant injury.

Acknowledgment—We appreciate the help of Dr. Kamaljit Kaur in synthesizing LGE_2.

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